Furanone quorum-sensing inhibitors with potential as novel therapeutics against Pseudomonas aeruginosa

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Furanones; the Quorum Sensing Inhibitors as Potential Therapeutics against *Pseudomonas aeruginosa*

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Abstract

Microorganisms use quorum sensing (QS), a cell density dependent process, to communicate. This QS mode of interchange leads to the production of a variety of virulence factors, co-ordination of complex bacterial behaviours, such as swarming motility, degradation of host tissue and biofilm formation. QS is implicated in numerous human infections and, consequently, researchers have sought ways of effectively inhibiting the process in pathogenic bacteria. Two decades ago, furanones were the first class of chemical compounds identified as Pseudomonas aeruginosa QS inhibitors (QSIs). *P. aeruginosa* is a ubiquitous organism, capable of causing a wide range of infections in humans, including eye and ear infections, wound infections and potentially fatal bacteraemia and thus novel treatments against this organism are greatly needed. This review provides a brief background on QS and the use of furanones as QSIs. Based on the effectiveness of action, both *in vivo* and *in vitro,* we will explore the use of furanones as potential antimicrobial therapeutics and conclude with open questions.

Introduction

Quorum sensing (QS) is a cell density-dependent method of bacterial communication and co-ordination, which is seen in many species, including *P. aeruginosa, V. fischeri* and *E. carotovora* [1]. QS systems in these organisms are often associated with altered phenotypes [2] and the regulation of complex processes such as biofilm formation [3–7], swarming motility [8] and virulence gene expression including those coding for degradative enzymes [9]. In brief, quorum sensing bacteria constitutively express low molecular weight signalling molecules known as autoinducers. In gram-negative organisms, the primary autoinducers are a group of molecules known as the N-acyl homoserine lactones (AHL)[10]. These AHLs are produced and released by bacteria and accumulate in their extracelluar environment. Once a critical environmental AHL concentration is reached, the signalling molecules will interact with their cognate receptors and induce the expression of their target genes. The regulation of these target genes can result in the production of molecules, such as endotoxins and degradative enzymes [11–13] or down regulation of surface antigens, such as OprH [14] to avoid detection by a host's immune system. Bacteria often have multiple QS systems, which work together in order to govern the organism's virulence and behaviour. A good example of this is the model gram-negative organism *P. aeruginosa,* which utilises 3 distinct quorum sensing systems known as the Las, Rhl, PQS systems and one recently proposed system, the IQS system. [15] . The Las QS system is responsible for the production of many virulence factors including the LasA, LasB, and Apr protease enzymes [16, 17]. While the Las QS system is only one QS system used by *P. aeruginosa*, it is typically thought of as being at the top of the QS hierarchy, primarily due to the fact that activation of the Las system causes a subsequent activation of both the Rhl and PQS systems[18, 19]. Activation of the Rhl QS system governs the transcription of a distinct set of virulence associated genes, such as those

involved in rhamnolipid, pyocyanin, and hydrogen cyanide production [20, 21]. As is the case with the Las quorum sensing system, the Rhl QS system is not wholly independent and it can be activated by the Las system, but can also supress the activation of the PQS system. This interrelation of the various QS systems allows *P. aeruginosa* to compensate for the loss or inhibition of one QS system and still remain virulent. The complexity of bacterial QS and the ability of many species to compensate for the loss of one or more QS systems makes exploiting the inhibition of quorum sensing a particularly challenging task.

An overview of the QS process in *P. aeruginosa* is presented in Figure 1 For a comprehensive overview of bacterial quorum sensing, the reader is referred to two recent reviews [22, 23]

Figure 1. Interrelation of the 3 primary quorum sensing systems (Las, Rhl and PQS) in the model gram-negative microorganism *Pseudomonas aeruginosa*. 1. Octododecanoyl homoserine lactone (OdDHL) accumulates in the extracellular environment and once a critical threshold is reached it diffuses back into the cell. 2. Here, the LasR interacts with, and causes dimerization of, the transcriptional activator, LasR . 3-4. The LasR/OdDHL complex then initiates transcription of numerous genes including *lasI* (thus producing more OdDHL), *lasA (*LasA protease*)* and *lasB* (LasB elastase*). These products then either diffuse out or are actively effluxed from the cell. 5. The LasR/OdDHL complex also activates transcription of the *rhlR and rhlI* genes. 6. Transcription of *rhlR* results in the production of the transcriptional regulator protein RhlR and transcription of *rhlI* results in production of Butyryl homoserine lactone (BHL). 7. BHL diffuses from the cell and accumulates in the extracellular environment resulting in a second positive feedback loop. 8. As with OdDHL, BHL enters the cell and binds with RhlR to cause a conformational change. The RhlR/BHL complex then promotes the transcription of *rhlI* leading to an increased production of BHL as well as the transcription of genes causing production of rhamnolipids and pyocyanin. 9. The RhlR/BHR complex also plays a role in negatively regulating the PQS system. 10-12. Finally, the PQS quorum sensing system, responsible for the production of pyocyanin and hydrogen cyanide is activated by the LasR/OdDHL complex causing the transcription of *pqsH* gene that results in the production of the *Pseudomonas* quinolone signal (PQS) [24]**. 13-14. PQS, in conjunction with the transcriptional regulator, PqsR, activates transcription of the *pqsABCDE* operon, positively regulating further transcription of *rhlR* and, *via* the *pqsABCDE* gene products, increasing expression of genes associated with production of hydrogen cyanide, rhamnolipids, biofilm and pyocyanin.**

QS is important not only for bacterial survival but also in allowing bacteria to colonise and infect a host by, for example, promoting bacterial adhesion to host cells [25] Additionally, QS–controlled behaviours, such as biofilm formation and virulence factor production, have been implicated in numerous health conditions including cystic fibrosis, development of chronic wounds and colonisation/infection of medical implants [26–28]. Biofilms, in particular are highly problematic in these conditions.

Biofilm formation is a process by which planktonic cells form organised communities. They are encased in a self–produced polymeric substance, often attached to a biotic or abiotic surface [29]. Briefly, biofilms are formed when planktonic bacteria attach, first reversibly then irreversibly, to a surface. This attachment results in changes in the expression of multiple genes involved in the biofilm formation process, such as the genes involved in the production of the polymeric matrix that provides the biofilm structure and some virulence factors [30, 31]. Bacterial cells begin to aggregate and form microcolonies, encased in the extracellular polymeric substance. These polymers are continually produced and gradually accumulate, while bacterial cells divide providing much of the three–dimensional structure of the biofilm. Finally, once the biofilm has matured, large quantities of surfactants, such as rhamnolipid, are produced. These surfactants allow the biofilm to disperse, thus returning some of its cells to a planktonic state [32]. Cells that have become planktonic again are then free to relocate and seed new biofilms. This dispersal may be brought about through the mature biofilm experiencing a damaging mechanical force or it may be triggered by environmental cues, such as an abundance of nutrients [33].

The role of QS in biofilm formation has been debated for some time with many published results providing contradictory findings. Despite this, QS has been implicated in many stages

of biofilm development in *P. aeruginosa*. For example, QS–controlled twitching motility is involved in the formation of the microcolonies in the early stages of biofilm formation[34]. As well as having a role in microcolony formation through the regulation of twitching motility, QS has also been shown to be involved in the process of biofilm maturation and the formation and maintenance of its three–dimensional structure [32], thus making QS an attractive target for new therapies.

One method of ameliorating the negative effect of processes, such as biofilm formation, is to inhibit the QS processes that control them, using compounds known as quorum sensing inhibitors (QSI). QSI include a range of natural and synthetic compounds that prevent autoinducers from binding to their cognate receptors, suppress the production of new autoinducers, or enzymatically degrade autoinducers before they are detected (also known as quorum quenching). In this review, we will focus on the potential utility of furanones as QSI. For a more general review of quorum sensing inhibitors, particularly their use as antibiofilm agents, the reader is referred to the recent work of Brackman and Coenye [35].

Furanones

Furanones are a family of structurally related molecules characterised by the presence of a 5 membered heterocyclic furan ring. Furanones are common in nature and are found in a wide variety of marine and terrestrial plants, including the red marine macroalga *Delisia pulchra*, strawberries and coffee [36, 37]. Some furanones are produced as a by-product of cooking food, for example in the Maillard reaction when baking bread, where they also act as flavour compounds [38, 39]. Furanones are also found in a number of fermented food products, including wine and Japanese soy sauce [40]. One of the final natural sources of furanones are

147 fungi and species, such as *Aspergillus spp.* They are capable of producing furanone derivative
148 compounds [41], which represent a potential new avenue of enquiry into QS inhibition.

149 As well as being present in a number of natural sources, furanones can be chemically
150 synthesised. Furanone derivatives have been chemically synthesised since the 1980s and
151 synthesis often began with relatively simple compounds, such as ketones and dimethyl
152 acetals or other simple organic precursors [42, 43]. Alternatively, modifications can be made
153 to existing furanone compounds, as well as additions to existing furanone structures as will
154 be discussed later in this review. Both natural and synthetic furanones have been shown to
155 effectively inhibit QS but synthetic furanones offer the possibility of precise control over
156 compound structure and, therefore, control of any potential off–target effects.

157 ## Efficacy of furanones against non-human pathogens

158 One of the earliest natural furanones to be discovered and characterised was 2,5-dimethy-4-
159 hydroxy-3(2H)-furanone. It is found in pineapple and was reported in 1965 by Rodin *et al.*
160 [44]. Using gas chromatography and nuclear magnetic resonance spectroscopy, they
161 determined that this aromatic compound must contain a cyclic five–membered ring structure
162 in addition to an arrangement of side groups that the authors were unable to definitively
163 characterise, despite similarities to a previously synthesised compound tetrahydro-3,4,-
164 furandione [44, 45]. In spite of the characterisation of this and other furanone compounds,
165 the structural similarities between furanones and gram-negative QS molecules was not
166 realised until after the identification of autoinduction by Nealson *et al.* in 1970 and the
167 structural characterisation of the chemical autoinducers (now known as N-acyl homoserine
168 lactones or AHL) in *Photobacterium fischeri* by Eberhard *et al.* in 1981 [46, 47]. The *P. fischeri*
169 autoinducer used to control luciferase production consisted of a five–membered ring with a

six-carbon chain and was identified as N-(3-oxo-hexanoyl)-3-aminodihydro-2(3H)-furanone. Although the structure of furanones and AHL had both been known for more than a decade at this point, investigations into their anti–quorum sensing properties began only in 1993 following the discovery of halogenated furanones in the marine alga *Delisea pulchra* [36]. As with the naturally occurring fruit furanones, these natural halogenated furanones were structurally similar to AHL. The similarity of the five membered ring structure found in both furanones and AHL, along with the ability of *D. pulchra* to resist epiphytic colonisation, prompted studies into the effects of these seaweed–derived furanones on AHL–mediated bacterial behaviours. Some of the first experiments showed furanone treatment greatly reduced *Serratia liquifaciens* swarming motility, as well as *Vibrio* spp. bioluminescence (up to 100–fold) [48], providing evidence that furanones could modulate QS–mediated behaviours.

Further investigation of QSI revealed that seaweed–derived furanones interfered with AHL mediated processes via displacement of the AHL signalling molecule from its cognate receptor. Using tritium–labelled AHL, Manefield *et al.* [49] showed that the addition of furanone to cultures of *Escherichia coli pHK724* (overexpressing a recombinant LuxR AHL receptor protein) resulted in a dose–dependent decrease in the proportion of labelled AHL in the cells when compared to untreated cells. They also showed that addition of two different,although unnamed, furanones to a culture of *E. coli* pHK724 that was already saturated with 0.5 μM exogenous autoinducer reduced the proportion of autoinducer bound to the LuxR receptor by over 75%. Two–dimensional gel electrophoresis then revealed that protein synthesis in furanone–treated cells was largely unaffected, save for reductions in the abundance of several Lux QS system proteins tentatively identified as LuxA, LuxB and LuxD, as well as OmpF, DnaK and Glutamate-ammonia ligase.

It has been shown by Welch *et al*. (2000) and Manfield *et al*. (2001) that, in *Erwinia carotovora*, treatment with an unnamed, seaweed derived, halogenated furanone (30 µM) reduced the secretion of virulence factors, including protease and cellulase. Furthermore, a dose–dependent response was observed between 10 µM and 40 µM, leading to a reduction in carbapenem production that was suggested to result from furanone–mediated interference in the quorum sensing–regulated production of the CarA and CarC proteins required for carbapenem-3-carboxylic acid production[50–52].

These reports showed that not only can natural furanones effectively inhibit quorum sensing, but that they could, in theory, be used to attenuate the virulence of some microorganisms. This leads logically to consideration of the potential of furanones in the battle against human pathogens.

Efficacy of furanones against human pathogens

In recent years, the majority of research into furanone–mediated QSI has focused primarily on the effects of these compounds on human pathogens and, in particular, the model organisms *E. coli* and *Pseudomonas aeruginosa*.

Efficacy of furanones against *Escherichia coli*

A natural furanone, known as (5Z)-4-bromo-5-(bromoethylene)-3-butyl-2(5H)-furanone (Figure 3), was demonstrated to inhibit bioluminescence in the marine organism *Vibrio harveyi* at concentrations between 16.13 µM and 32.26 µM. The same furanone also attenuated biofilm formation in *E. coli,* reducing average biofilm thickness by 55% and the number of viable cells by 87 % at a concentration of 164 µM. Additionally, lower furanone concentrations (64.5 µM) significantly reduced *E. coli* swarming motility [53]. This important work clearly demonstrated that furanones could interfere with QS processes and that the

phenomenon could be used to combat virulence in human pathogens. Investigations into the effects of synthetic brominated furanones showed that these compounds could also reduce *E. coli* biofilm thickness and surface coverage by up to 50% [54] and that, when applied at 50 µM, could inhibit swarming motility and reduce biofilm production by up to 40 % in the food–borne pathogen *E.coli* 0103: H2 [55, 56].

Efficacy of furanones against *Pseudomonas aeruginosa*

Natural furanones greatly reduce the production of *P. aeruginosa* virulence factors including protease (up to 43 %), chitinase and pyoverdine (by almost 100%) [57–59]. However, it becomes clear that the efficacy, and effect, of furanones in decreasing bacterial virulence may not be consistent. For example, the near total prevention of pyoverdine production by C-30, a synthetic derivative (Figure 3) of the natural seaweed–derived furanone, was reported by Hentzer *et al.* in 2003 [58]. The later work of Ren *et al.* (2005), however, demonstrated that both *P. aeruginosa* PAO1 and JB2 exhibited an increase in siderophore production when exposed to the natural furanone (5Z)-4-bromo-5-(bromoethylene)-3-butyl-2(5H)-furanone at concentrations between 20 µg mL^{-1} and 100 µg mL^{-1} (64.5–322.5 µM) [60]. This discrepancy is most likely due to the differences in structures of the furanone used. The natural furanone used by Ren *et al.* has a hydrocarbon chain bound to the furanone ring [61], whereas this is substituted for a single hydrogen in the synthetic furanone, C-30 [58] (Figure 3). Nonetheless, the observed differences in effects mediated by these two molecules suggest that alterations in the chemical structure of furanones or their derivatives result in significant differences in their QSI activity.

Figure 3. Comparison of natural and synthetic furanone structures: (A) naturally occurring (seaweed–derived) furanone (5Z)-4-bromo-5-(bromoethylene)-3-butyl-2(5H)-furanone, (B) the synthetic furanone known as C-30. Both compounds exhibit similarities in structure, consisting primarily of a five–membered furan ring, however, C-30 lacks a hydrocarbon

chain. The compounds have different biological activities, leading to the conclusion that changes in side group structure play a key role in determining furanone compound efficacy as QSI.

In addition to their ability to reduce virulence factor production in a range of microorganisms, one of the most important effects of furanone compounds on *P. aeruginosa* is their ability to interfere with biofilm formation. Investigations in the early 2000s showed that two synthetic furanone derivatives in particular, C-56 and C-30, were highly effective in inhibiting *P. aeruginosa* biofilm formation [57, 58]. These furanones interfered with quorum sensing in both planktonic cultures and, more importantly, in established biofilms. While furanone C-56 did not appear to affect the initial bacterial attachment stage of biofilm formation, cells treated with 5 μg mL^{-1} (28.5 μM) C-56 developed significantly thinner biofilms, exhibiting an approximate 37% reduction in overall thickness. The synthetic furanone–treated biofilms also appeared to be more sparsely populated than untreated biofilms and overall the data suggested that furanones might have a significant long–term effect on both biofilm formation and maintenance. This idea was further supported by the recent work of Choi *et al.* in 2014 [59] who showed that, in PAO1, treatment with a natural furanone 4-hydroxy-2,5-dimethyl-3(2H)–furanone (HDMF) reduced rhamnolipid production. Rhamnolipids are essential for maintenance of biofilm structure and integrity [32, 59] and PAO1 cells grown in the presence of either 0.1 μM or 1 μM HDMF formed biofilms, but with reduced biofilm biomass (27.8% and 42.6%, respectively). Treatment of 48 hour PAO1 biofilms with either 0.1 μM or 1 μM HDMF also led to subsequent 66.3% and 84.8% losses, respectively, of biofilm biomass over the following 48 hours, whereas untreated biofilms lost only ~50% of their biomass in the same time period. This data suggests that HDMF treatment accelerates the natural, post 48 hour, loss of biomass observed in mature *P. aeruginosa* biofilms. Biofilm morphology was also

changed, as furanone–treated late–stage biofilms presented as poorly populated monolayers in comparison to the more natural 3D architecture and dense cell population of the untreated controls.

Kim *et al.* (2008) showed that a synthetic furanone compound, named 5a, could efficiently reduce the initial attachment of planktonic *P. aeruginosa* cells to glass slides [62]. While no mechanism for the phenomenon was reported by the authors, the differences observed suggest that, although different furanones may inhibit QS and biofilm production, they may do so in quite different ways, possibly by acting upon different elements of the QS–mediated processes that lead to biofilm formation.

Numerous other furanone compounds can also effectively reduce biofilm formation in PAO1. Reductions of up to 43 % were shown by Shetye *et al*. [54] using a range of synthetic brominated furanones. Four of these in particular were found to be effective in reducing either *E. coli* or *P. aeruginosa* biofilm formation. Despite their apparent efficacy, they have not been characterised as thoroughly as C-30 and C-56.

Furanone compounds have another important effect on *P. aeruginosa*, specifically on so–called "persister cells"–a type of largely dormant and consequently less antibiotic–sensitive cell found in biofilms. Persister cells are believed to be involved in maintaining chronic and recurrent infections, particularly in conditions such as cystic fibrosis [63, 64]. Certain furanones, however, can sensitise PAO1 persister cells to antibiotics, reducing their numbers and making biofilm eradication more likely. Treatment with between 5 μg mL^{-1} and 100 μg mL^{-1} of a synthetic brominated furanone, (Z-4-bromo-5-(bromoethylene-3-methylfurn-2(5H)-one, resulted in a dose–dependent reduction in planktonic PAO1 persister cell numbers from over 1×10^5 CFU mL^{-1} to approximately 1×10^4 CFU mL^{-1} [65]. Interestingly, while the number

289 of persister cells was reduced, total cell numbers were not affected by the furanone
290 treatment. Importantly, this reduction in persister cells also occurred in surface–attached
291 biofilms, and here, a 2 $\log_{10}$ reduction in numbers from 1×10^4 CFU mL^{-1} to 1×10^2 CFU mL^{-1}
292 was observed following treatment with 60 μg mL^{-1} of the synthetic brominated furanone. In
293 addition, a relatively low dose of furanone also increased the efficacy of tobramycin and
294 ciprofloxacin in killing persister cells: 5 μg mL^{-1} of synthetic brominated furanone followed by
295 tobramycin treatment reduced persister cell numbers by over 99.9 %, an order of magnitude
296 greater than antibiotic alone, while treatment with ciprofloxacin reduced persister cell
297 numbers by approximately 90 % compared to a 50 % reduction with antibiotic only. Thus,
298 furanone addition potentiated biofilm persister cell killing by reducing the antibiotic tolerance
299 of the cells. Two further, structurally distinct, furanone compounds also led to a reduction in
300 persister cell numbers. However, it was also observed that several other distinct furanones,
301 while capable QSI, had no effect on persister cells [65, 66]. As these furanones were all
302 structurally related, it seems likely that differences in activity were dictated by small changes
303 in chemical structure. The observation that two non-brominated compounds (named NF1 and
304 NF2), which despite having structural similarity to (Z-4-bromo-5-(bromoethylene-3-
305 methylfurn-2(5H)-one had no effect on either QSI activity or persister cells, suggests that
306 bromine, specifically, is important for activity against persister cells. However, it should be
307 noted that these non-brominated furanones did not only differ from the brominated
308 furanone in their lack of halogen groups, but also in the abundance and position of other side
309 groups. For example, the non-brominated furanone NF2 contained a methoxy group, which
310 was not present in the brominated furanone. Additionally, furanone NF1 had an additional
311 carbonyl group, which is not present on the brominated furanone. These changes will have
312 undoubtedly affected the furanones reactivity and played a role in the difference in bioactivity

313 seen by Pan *et al.* (2013). Due to these differences, firm conclusions cannot be made
314 regarding the effect of bromination and furanone activity against persister cells [66].

315 ## Mechanism of furanone action

316 Furanone concentrations of up to 10 µg mL^{-1} have been demonstrated to reduce expression
317 of the *lasB* gene that encodes the LasB elastase, resulting in an approximately 50 % reduction
318 in relative fluorescence of a *PlasB-GFP* reporter [54, 57]. This suppression was non-reversible,
319 even following addition of moderate levels of exogenous autoinducer. Addition of 80 nM
320 OdDHL and 2 µg mL^{-1} of C-56 activated *lasB* expression, while the addition of 40 nM OdDHL
321 and 2 µg mL^{-1} C-56 did not. This elegantly demonstrates that the furanone binds competitively
322 to the native AHL receptor. The possibility of C-56 acting against any other part of the QS
323 network was ruled out by use of *E. coli* MT102, which lacks any AHL–mediated QS system
324 other than the recombinant *PlasB-GFP* fusion [57]. Reductions in C-56–mediated *lasB*
325 expression were also observed in established biofilm as well as in planktonic cultures. Overall,
326 the experiments suggested that the furanone's antagonistic activity stems from competition
327 with the native autoinducer to bind to, and subsequently block, the AHL receptors.

328 Gene expression studies carried out by Hentzer *et al.* [58] in PAO1 showed that expression of
329 85 out of 93 differentially expressed genes was reduced following treatment with furanone
330 C-30. A large proportion of these genes had previously been described as QS–controlled,
331 including *lasA*, *lasB* and several other genes involved in virulence factor production.
332 Interestingly, it was shown that expression of *fabH1* and *fabH2*, encoding subunits of the 3-
333 oxo-acyl carrier protein (ACP) synthase III which has been suggested to be involved in the
334 production of AHL [10, 67], was considerably reduced (~50 and 80–fold, respectively). This
335 suggested that furanones could inhibit QS by reducing the quantity of ACP transcripts, leading

to decreased synthesis of AHL molecules. Genes exhibiting increased expression following C-30 treatment included those of the *mexEF-oprN* operon that encodes components of a multidrug efflux pump [68] and genes encoding ATP-binding cassette (ABC) transporters and major facilitator superfamily (MFS) transporters. These transporter proteins enable both influx of nutrients and the active removal of toxins from bacterial cells [69, 70] and their increased expression may be due to the tendency of bacteria to increase efflux activity in the presence of antimicrobial compounds [71]. Interestingly, expression of *mexR*, the repressor for the multidrug resistance efflux pump encoded by *mexAB-oprM*, exhibited increased expression (~5–fold) suggesting that, in the presence of the furanone, *P. aeruginosa* could be resensitised to certain antibiotics normally excreted by this system. The expression of the other *mex* efflux operon, *mexEF-oprN* (induced by nitrosative stress and chloramphenicol [68]) was, however, increased by furanone treatment (>5–fold) and may be consistent with the cells attempting to actively remove the intracellular furanone. Hentzer *et al.* also showed that although C-30 did not prevent PAO1 biofilm formation, it exerted a significant effect on the sensitivity of 72 hour biofilms to 10 µM tobramycin as demonstrated *via* BacLight staining [58]. The possibility of furanone–mediated re–sensitisation of PAO1 to antibiotics was not, however, further investigated by the authors.

This reduction in QS–associated gene expression does not appear to be universal for all QS systems. The work of Shetye *et al*. on the *P. aeruginosa* Rhl QS system utilised *E. coli* containing a P*rhlI-LVA-GFP* reporter plasmid. Treatment with 100 µM to 200 µM of either of two synthetic brominated furanones caused an increase in fluorescence intensity (caused by increased transcription of the *rhlI-LVA-GFP* genes) rather than an antagonistic effect. Thus, treatment with 200 µM furanone BF15 more than doubled transcription of the P*rhlI-LVA-GFP* fusion, in contrast to the effect on a P*lasI-LVA-GFP* reporter for which fluorescence was

360 reduced by approximately 50 % by the same treatment [54]. This observation provides further
361 evidence that furanone action on QS systems is highly variable, reinforcing the idea that their
362 effects may not always be antagonistic. The authors hypothesised that, in the light of similar
363 effects caused by minor structural changes to native signalling molecules, a low binding
364 constant between the synthetic furanones and their cognate receptors could explain their
365 observations. This hypothesis is further supported by data from molecular docking studies–
366 an important tool in the process of novel drug discovery and characterisation [72] and which
367 have been used to good effect in the search for bioactive furanone compounds. Liu *et al.*
368 (2012) demonstrated that furanones associate with the LasR receptor protein in a number of
369 ways depending on the structure of the given furanone. Synthetic furanones generally
370 associate with the LasR protein *via* hydrogen bonding between the carbonyl group on the
371 furan ring and the amine functional group of Arg 61 in the LasR receptor protein. Several
372 other potential furanone–receptor binding sites were identified *in silico* within both the
373 receptor protein itself and also in furanone molecules. However, these were unique to the
374 furanones tested [73]. This work clearly shows that the affinity of a furanone for a receptor
375 protein is likely to be highly dependent on furanone structure. This further supports the
376 hypothesis put forward by Shetye *et al.* [54] that reduced affinity for the receptor may have
377 caused the agonistic activity of their synthetic furanone lacking a hydrocarbon chain. These
378 studies provide evidence that while many furanones may show antagonistic behaviour
379 towards *P. aeruginosa* QS systems, it is by no means a universal rule.

380 Further evidence for the importance of furanone structure and its relationship to biological
381 effect was provided by Brackman *et al*. in 2012 [74]. They demonstrated that a group of
382 synthetic AHL derivatives known as triazolyldihydrofuranones, in which the amide function is
383 replaced by a triazole group, displayed a degree of anti–QS activity as measured using a QSIS2

biosensor strain of *P. aeruginosa*. The furanones' activity was dependent on the length of the carbon chain bound to the furan ring and compounds with C_{10} or C_{12} chains inhibited OdDHL–mediated QS most effectively. It was suggested that this was due to the similar side chain length (C_{12}) found on the native signalling molecule and indeed the effect of chain length has also been highlighted in examinations of QSI with other AHL derivatives [75, 76]. It is important to note that the primary AHL molecules used in Lux, Las and Rhl QS systems all have carbon chains attached to their five–membered ring and it may follow that the closer the furanone structure is to the native AHL for the system, the more effectively it can inhibit that particular QS system. This hypothesis is supported by the work of Ahumendo Monterrossa *et al.* [77] who showed that the location and orientation of the AHL side chain within the LasR receptor is correlated with the biological activity of the ligand. Changes in ligand functional groups result in agonistic or antagonistic changes in ligand effect, with the interaction between the AHL acyl side chain and Tyr 47 in LasR dictating the stability of the resultant AHL–LasR complex. The lack of a side chain similar to that of the native AHL (for example, a shorter, or absent carbon chain) may result in a furanone not complexing as well with the LasR receptor, leading to a less potent effect [77].

While many studies have focused on the synthesis and testing of furanone compounds with different structures, such as the work of Chang *et al.* (2019) [78], the compounds in these studies often differ in more than one structure and it is difficult to ascertain which structural moiety is impacting on the QSI capability of the molecule. It is also often the case that studies in which large numbers of furanone derivatives do not extensively test the QSI potential of the derivatives with many using decreases in biofilm formation (as measured by a simple crystal violet assay) as a proxy for QSI. When these derivatives are tested more thoroughly it

is most often the most effective compounds and no direct comparison can be made between those moieties that increase efficacy and those that do not.

It is clear that the structure of a furanone derivative has an impact on the QSI efficacy of the compound. Unfortunately, no concerted effort to elucidate the exact structure function relationship of various side groups and, thus, further research is needed.

Although, the structure of a given furanone undoubtedly has a significant effect on the bioactivity of the compound, the structure of the target receptor protein will also have an impact on the QSI ability. Gram negative bacteria typically employ two types of QS receptor proteins, namely transmembrane, two component, LuxN type receptors and the cytoplasmic LuxR family of receptors, which typically consist of an AHL binding domain and a DNA binding domain [79, 80]. There is a high degree of variability between the receptor proteins of different QS systems and even greater variability between receptors of different species. For example, while the LasR receptor protein of *P. aeruginosa* has a high structural similarity to the TraR receptor of *Agroacterium tumefaciens* and the SdiA protein from *E.coli* the amino acid sequence only sows a 10-16% similarity [81]. It likely that although these receptor proteins share similar topology the way in which they bind AHL and, therefore furanones, is likely quite different.

This variability in protein structure is also seen between receptor proteins from different QS systems from the same organisms. For example, despite both LasR and RhlR being from *P. aeruginosa*, both being activated by structurally similar AHL and thus being closely linked, RhlR (of which no structure and therefore no specific ligand binding data, has been elucidated) actually shares a higher sequence homology with the more distant SdiA receptor protein of *E.coli* [82]. Although their cognate receptors are similar, it appears that the

mechanisms of ligand binding are quite distinct. Taken together, data from a variety of molecular investigations suggest that the effects of furanones on QS systems is variable. It is clear that not only can furanones interact with a number of different QS systems, but also that they may do so with varying degrees of success and that, furthermore, this efficacy is influenced by a range of structural factors both in the structure of the furanone, including carbon chain length and the type of functional side groups found on the lactone ring and structural differences in the target receptor proteins.

Efficacy of furanones *in vivo*.

Considering the useful biological effects of furanone QS antagonists on *in vitro* systems, there is clear potential for their application to *in vivo* systems. However, there is a lack of data regarding their efficacy or safety *in vivo*, in addition to a certain amount of contradiction amongst the reports published to date. In 2003, Hentzer *et al.* showed that, in a mouse model of 48 h *P. aeruginosa* lung infection, the intravenous administration of the synthetic furanone C-30 at a concentration of 1.7 µg g^{-1} body weight resulted in a reduction in bacterial QS in the lung tissue, as measured *via* a *lasB-GFP* reporter. However, this inhibition was not permanent and QS recovered to pre–treatment levels after approximately 6 hours. Given the transient nature of the effect, further experiments showed that a subcutaneous dose of C-30 (0.7 µg g^{-1} body weight) administered every 8 hours for 3 days immediately following infection with PAO1 enabled the mice to better clear PAO1 from the lungs when compared to a vehicle control [58]. This was later confirmed by Wu *et al.* in 2004 [83] who demonstrated that intravenous administration of 17 µg g^{-1} body weight of the synthetic furanone C-56, 24 hours post–PAO1 infection, resulted in a significant reduction in QS in murine lung tissue as measured using a PAO1 *dsred-lasR-PlasB-GFP* reporter strain . As with C-30, this C-56–

453 mediated reduction was only temporary and QS levels returned to baseline values after
454 approximately 8 hours. Additionally, Wu *et al*. (2004) showed that oral–as opposed to
455 subcutaneous–administration of 5 μg g^{-1} body weight of C-56 resulted in a significant decrease
456 in both QS in the lung and in mouse mortality. Following infection with a lethal dose of PAO1
457 and treatment with 5 μg g^{-1} C-56 three times a day for 2 days, mouse survival increased from
458 12 % to 45 % at 48 hours post–infection. In contrast to the intravenous administration of C-
459 30 in the work of Hentzer *et al*. [58], Wu *et al.* used subcutaneous administration of between
460 0.25 μg g^{-1} and 0.7 μg g^{-1} C-30 three times a day for 3 days. This resulted in significantly better
461 bacterial clearance from the lung at 7 days post–infection in mice infected with sub-lethal
462 doses of PAO1, where 1000–fold lower lung CFU numbers resulted following C-30 treatment.
463 Although no survival rate data was provided for C-30 treated animals, this furanone was more
464 effective in assisting with bacterial lung clearance than furanone C-56. Furanone–treated
465 mice also exhibited less severe lung pathology, with fewer abscesses forming in the C-30
466 treated mice and less tissue damage in the C-56 group. These studies show that not only were
467 C-30 and C-56 effectively carried to the lungs following oral, intravenous and subcutaneous
468 administration, but also that they retained their biological activity. Thus, it is clear that both
469 C-30 and C-56 may be highly beneficial in combatting *P. aeruginosa* lung infections *in vivo.*

470 There is evidence that, in a primary human airway epithelial cell infection model, the natural
471 furanone, HDMF, may be beneficial. It is known that *P. aeruginosa* diffusible virulence factors
472 (e.g. LasA protease, LasB elastase) impair several important functions in airway epithelial cells
473 including wound healing and cell proliferation and migration [84]. However, in 2016 Ruffin *et*
474 *al.* [85]showed that growth of PAO1 in media supplemented with 125 μg mL^{-1} HDMF led to
475 reduced secretion of virulence factors, highlighted by a 70 % decrease in elastase activity
476 measured using an elastin–congo red assay. It was also observed that wound healing rates in

epithelial cell scratch assays were significantly better when exposed to diffusible materials from HDMF–treated PAO1 cells than when exposed to the secretions from untreated PAO1 cells. Diffusible materials from non-furanone-treated PAO1 reduced the wound repair rate by ~50 %, while repair rates for cells exposed to diffusible material from HDMF–treated PAO1 cells was not significantly different from the controls. This would suggest that HDMF treatment decreased QS, resulting in a decrease in virulence factor production leading to better outcomes in wound repair. A similar result was obtained with highly differentiated airway epithelial cells, indicating the possible utility of HDMF in the treatment of lung infections, especially given that exposure to HDMF alone did not have a negative impact on the wound healing capacity of airway epithelial cells [85]. While HDMF apparently reduces bacterial virulence factor secretion, it is unfortunate that, to date, no data have been published regarding the cellular toxicity of HDMF. However, it could be hypothesised that because wound repair rates were not negatively impacted by HDMF, that cell viability also was not significantly impacted. Nonetheless, further investigation into the cytotoxicity of HDMF is needed to confirm this.

Furanones as potential therapeutics

The published evidence for the efficacy of furanones in murine models is somewhat limited, but together with the *in vitro* human cell data, it suggests that these compounds have potential as effective human therapeutics. A number of studies have attempted to use furanones in a clinically relevant way. For example, in 2012 Kim *et al*. demonstrated that co–administration of furanone with ciprofloxacin significantly reduced *in vitro* biofilm formation using a medical implant as a substratum. The dual administration of 50 µg mL^{-1} 5-hydroxy-2(3H)-benzofuranone and 1 µg mL^{-1} ciprofloxacin significantly reduced numbers of planktonic

cells and also effectively prevented *P. aeruginosa* biofilms from forming on silicone tympanostomy tubes. While the combination therapy prevented biofilm formation, treatment with the furanone only led to a reduced level of biofilm formation without a simultaneous reduction in planktonic cell numbers[26]. Thus, the presence of the furanone appeared to inhibit biofilm effectively–most likely by interfering with QS signalling– allowing the antibiotic unrestricted access to planktonic cells. It is likely that when furanone treatment is stopped, however, that planktonic cells will then begin to form biofilms once again, making dual treatment necessary.

Siebert *et al*. (2016) recently suggested that chemical conjugation of furanones to other drugs currently in use might facilitate development of single molecule, multiple effect treatments. For example, a synthetic brominated 2(5H) furanone was joined with rosiglitazone, a common antidiabetic drug also known to have anti-inflammatory properties [86]. The furanone was linked to a rosiglitazone skeleton in an attempt to combine the QSI properties of the furanone with the anti-inflammatory properties of rosiglitazone. This combination of effects could be very useful in treating chronic infections, such as those found in wounds, where biofilm formation often causes a prolonged inflammatory state [87]. Xu *et al.* (2018) showed that treatment with 10 µM rosiglitazone–furanone fusion inhibited QS by up to 50% across the Las, Rhl and PQS quorum sensing systems as shown by a reduction in fluorescence of PAO1 *lasB-GFP*, *rhlA-GFP* and *pqsA-GFP* reporter strains, respectively. In addition they observed a reduction in the pro–inflammatory cytokines Tissue Necrosis Factor–α (TNF-α) and Interleukin–6 (IL-6)in RAW264.7 murine macrophages, showing that the rosiglitazone–furanone compound simultaneously inhibited QS and achieved anti-inflammatory effects [88]. Thus, while effective on their own, furanones have the potential to be combined with various other compounds to produce new, more effective therapies.

Bacterial resistance to furanones

One of the main reasons furanones are so attractive to researchers investigating quorum sensing inhibition is the fact that furanones do not significantly affect bacterial numbers and show no significant impact on bacterial growth kinetics when used in low doses [58, 89].

As with antibiotics, the issue of resistance to furanones, and quorum sensing inhibitors in general must always be considered. It has often been stated, when discussing the use of QSI compounds, that as inhibition of bacterial signalling may constitute a reduced evolutionary pressure and present less risk of resistance developing [58, 90, 91]. However, it has become apparent in recent years that this may not be the case. In 2012 Maeda *et al.* demonstrated that *P. aeruginosa* PA14 transposon mutants with a high resistance to furanone C-30 had disruptions in the in the *mexR* gene, which acts as a repressor of the mexAB-OprM operon. This mutation causes an overproduction of the MexAB-OprM efflux pump, suggesting a role for heightened efflux in resistance against this furanone [92]. This work was further supported by the work of García-Contreras *et al*. who, in 2013, assessed the effect of C-30 on virulence factor production in 50 clinical strains of *P. aeruginosa* isolated from cystic fibrosis patients. They found that while many clinical isolates responded to C-30 with greatly reduced elastase and pyocyanin production, several strains showed either no decrease in virulence factor production, or exhibited an increase in production of greater than 100 %. One strain in particular IP-42 exhibited significantly higher C-30 efflux capacity, while two other strains had significantly decreased C-30 uptake. While the reason for this decreased uptake is not known, the authors suggested a possible mutation in a transporter protein which they say “have not yet been identified” [93]. Despite many strains responding to C-30 as expected, with reductions in virulence factor expression, the authors suggested that furanones should be

used with caution in the clinical setting as many resistant strains may already exist, with–in extreme cases–the furanone compound exacerbating the infection.

Resistance may also arise through mutations and changes that do not occur in the quorum sensing system "circuit". It has been previously reported by Feltner *et al.* in 2016 that some clinical isolates of *P. aeruginosa* show variations in nucleotide sequence of the *lasR* gene of the Las QS system [94]. These isolates were obtained from cystic fibrosis respiratory cultures during the EPIC study [95], and when their *lasR* genes sequenced, 22% of the genes encoded a LasR protein that was significantly different to that of laboratory strains of *P. aeruginosa* also analysed. The mutations included insertions, deletions and single nucleotide polymorphisms (resulting in either amino acid changes or stop codons leading to truncated proteins). These LasR variants were either functionally different, or non-functional (LasR deficient). However, the most interesting finding of this study was that in the LasR deficient variants there was no significant change in a number of QS related phenotypes, such as motility or pyocyanin production. The authors then showed that the expression of some QS related phenotypes was maintained by the Rhl QS system in the absence of a functional LasR protein. This study demonstrated that clinical isolates can possess mutations that cause a 'rewiring' of the QS systems and their regulation. This presents a significant problem for the use of furanones, and other QSI, as a potential therapeutic as targeting a specific QS system may not be an effective treatment in such QS variants.

While resistance to furanones and QSI in general may be the result of repeated exposure, altered compound transport into and out of bacterial cells or mutations in the QS system genes themselves it is clear that the use of furanones may not be a universally effective treatment.

Outstanding questions

While many advances have been made in the use of furanones as QSIs and potential therapeutics several important questions remain unanswered. Firstly, do all furanones exert their QSI effects *via* the same mechanism and what effect does furanone structure have on the mode of action? Characterisation of structural indicators of efficacy using a furanone compound library and fluorescent QS reporter strains may help to develop a method for rapid identification of potentially useful furanone compounds or provide opportunities to modify current compounds to increase efficacy.

If furanones are to be used in humans as a therapeutic agent the effect of therapeutic concentrations of furanones on mammalian cells must be investigated. Further experimentation is needed to elucidate any deleterious effects on human cells in relation to protein production, cell proliferation and release of inflammatory mediators. Additionally, will the positive effects seen with *in vitro* studies and in the small number of mouse models translate to use in humans? If furanones are not shown to be detrimental to human cell lines, will their efficacy translate to systemic use or will drug activity be lost due to their metabolism in the liver or clearance by the kidneys?

While furanones are thought to be effective against pathogens at non-lethal concentrations, it is unclear if resistance to furanones can be avoided by using sub inhibitory concentrations? What are the potential mechanisms of furanone resistance? For example, would mutations in Las and Rhl receptors result in furanones not being able to bind effectively, or does increased efflux capacity allow the cells to better remove furanones? If resistance is unavoidable, are there methods by which the mechanisms of resistance could be subverted?

593 Another important consideration is the route of administration of these compounds. How
594 could furanones be administered effectively to combat infections in which quorum sensing
595 and quorum sensing controlled behaviours are a significant problem? Does method of
596 delivery significantly impact the efficacy of the compounds?

597 Finally, there is the issue of off target effects arising from furanone therapy. What, if any, are
598 the effects of topical and systemic use of quorum sensing inhibitors on the microbiome? It is
599 known that many microbial communities, such as those found on the skin and in the gut, are
600 dependent on effective quorum sensing and, to date, it is unclear what the effect of using
601 quorum sensing inhibitors such as furanones on these communities would be.

602

603 ## Concluding remarks

604 Quorum sensing is a method of cell–cell communication responsible for the production of
605 numerous virulence factors and the coordination of complex bacterial behaviours such as
606 biofilm formation. Quorum sensing and its related processes have been shown to be involved
607 in the formation and maintenance of a range of clinically relevant infections. In recent years
608 numerous compounds, including furanones, have been investigated for their ability to
609 interfere or inhibit the process of quorum sensing. Furanones have been demonstrated to
610 significantly decrease virulence factor production and biofilm formation in a range of
611 environmental and clinical pathogens. However, concerns have been raised surrounding both
612 the potentially negative effects of furanones on human cells and the possibility of developing
613 resistance. The material considered in this review clearly demonstrates that while both
614 natural and synthetic furanones have great potential as novel antimicrobial therapeutics,
615 there are a number of questions which still need to be addressed. More focused studies on

616 the efficacy of furanones as therapeutic agents in animal models are required as well as
617 research into novel delivery methods for the compounds themselves. Critically, investigations
618 are also needed regarding possible adverse effects on mammalian cells. If these issues are
619 addressed it is clear that furanones may prove to have excellent potential in combatting
620 bacterial infections in an age of widespread antimicrobial resistance.

621

Statement of Conflict

The authors note no conflict of interest.

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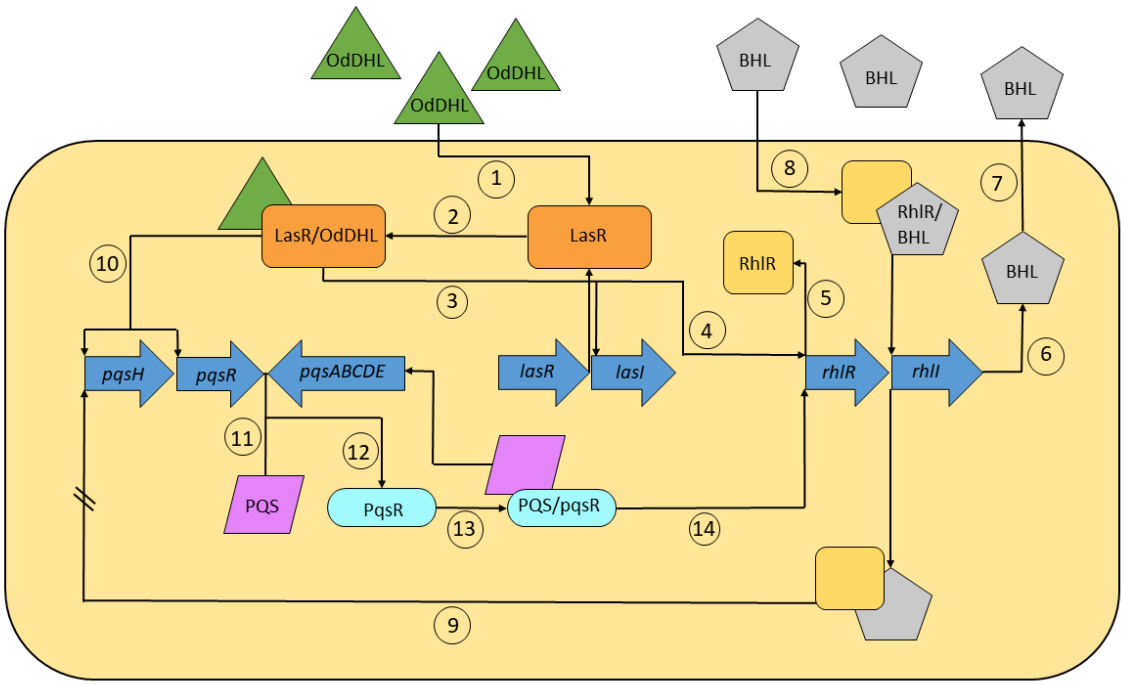
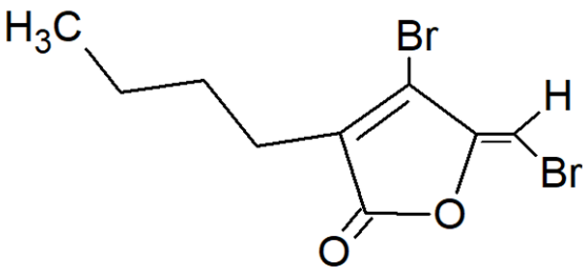
Figure 1



920 Figure 2

A



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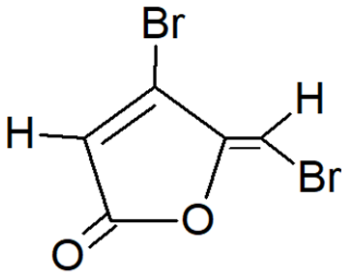

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